

Norrin, Frizzled-4, and Lrp5 Signaling in Endothelial Cells Controls a Genetic Program for Retinal Vascularization

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SUMMARY

Disorders of vascular structure and function play a central role in a wide variety of CNS diseases. Mutations in the Frizzled-4 (Fz4) receptor, Lrp5 coreceptor, or Norrin ligand cause retinal hypovascularization, but the mechanisms by which Norrin/Fz4/Lrp signaling controls vascular development have not been defined. Using mouse genetic and cell culture models, we show that loss of Fz4 signaling in endothelial cells causes defective vascular growth, which leads to chronic but reversible silencing of retinal neurons. Loss of Fz4 in all endothelial cells disrupts the blood brain barrier in the cerebellum, whereas excessive Fz4 signaling disrupts embryonic angiogenesis. Sox17, a transcription factor that is upregulated by Norrin/Fz4/Lrp signaling, plays a central role in inducing the angiogenic program controlled by Norrin/Fz4/Lrp. These experiments establish a cellular basis for retinal hypovascularization diseases due to insufficient Frizzled signaling, and they suggest a broader role for Frizzled signaling in vascular growth, remodeling, maintenance, and disease.

INTRODUCTION

The mature vasculature can be divided into arteries, veins, and capillaries, and vascular cells into endothelial cells (ECs) and mural cells (MCs), with additional anatomic, cellular, and molecular diversity in each of these categories. The vasculature is generated by precisely orchestrated patterns of vasculogenesis and/or angiogenesis followed by remodeling and stabilization concurrent with the establishment of EC-MC interactions (Armulik et al., 2005). In keeping with the complexity of vascular development

and structure, multiple cell signaling systems—including the VEGF, Netrin, Ephrin, Notch, and Angiopoietin systems—have been implicated in coordinating the proliferation, migration, adhesion, and differentiation of vascular cells (Adams and Alitalo, 2007).

The present study focuses on the retinal vasculature. This vasculature plays a central role in a variety of ocular diseases, including diabetic retinopathy and retinopathy of prematurity (Gariano and Gardner, 2005). The retinal vasculature presents a simple and stereotyped architecture. The major arteries and veins reside on the vitreal face of the retina and are oriented radially from their origin at the optic disc. They give rise to a series of smaller vessels that penetrate the retina and connect to a pair of planar capillary beds flanking a central layer of neurons (the inner nuclear layer). In the mouse, as in other mammals, the initial phase of retinal vascular development is characterized by radial growth from the optic disc, followed by the development of the intraretinal capillaries.

An unusual signaling system with profound effects on retinal vascular development has emerged from the study of Norrie disease, familial exudative vitreoretinopathy (FEVR), and osteoporosis-pseudoglioma syndrome (Berger and Ropers, 2001; Robitaille et al., 2002; Jiao et al., 2004; Toomes et al., 2004; Ai et al., 2005). In these genetic diseases, retinal hypovascularization is caused by partial or complete loss-of-function mutations in the genes coding for the cystine knot protein Norrin, the integral membrane receptor Frizzled-4 (Fz4), or the Fz4 coreceptor Lrp5, respectively. The similar retinal hypovascularization phenotypes observed in *Ndp* (Norrie disease protein), *Fz4*, or *Lrp5* loss-of-function mouse models, the high affinity and specificity of Norrin-Fz4 binding, and the potent activation of canonical Wnt signaling following coexpression of Norrin, Fz4, and Lrp5 imply that Norrin is an in vivo ligand for Fz4 (Kato et al., 2002; Xu et al., 2004; Richter et al., 1998; Luhmann et al., 2005b; Smallwood et al., 2007; Xia et al., 2008).

These observations raise a series of interrelated questions that are central to understanding the cellular basis and functional

significance of Norrin/Fz4/Lrp signaling. Is the primary site of Fz4 signaling—and hence the primary cellular defect in inherited diseases in which this system is impaired—neuronal, glial, or vascular? At the physiological and behavioral levels, what aspects of visual function are impaired by vascular defects that result from loss of Fz4 signaling? What cellular processes in vascular development require Fz4 signaling in the intact animal and in cell culture? Is Fz4 signaling a general feature of vascular development outside of the retina? Does Fz4 signaling alter gene expression, and, if so, how does this relate to vascular development? In the present work, we have addressed these questions by studying the effects of gain and/or loss of Fz4 signaling in vivo and in cell culture.

RESULTS

An Essential Role for Fz4 in Retinal Endothelial Cells

To assess the pattern of Fz4 expression and to permit cell type-specific deletion of Fz4 while simultaneously marking cells, we generated the Fz4 conditional knockout (CKO) allele *Fz4^{CKOAP}* (Figure 1A). In the absence of Cre-mediated recombination, *Fz4^{CKOAP}* functions as a wild-type (WT) allele and the distal alkaline phosphatase (AP) is not expressed. Cre-mediated recombination excises the Fz4 coding and 3' untranslated region (UTR) and places AP under the control of the Fz4 promoter. Thus, Cre-mediated recombination converts *Fz4^{CKOAP/+}* cells into phenotypically WT *Fz4^{AP/+}* cells. When *Fz4^{CKOAP}* is in combination with a conventional Fz4 knockout allele (Wang et al., 2001), Cre-mediated recombination converts phenotypically WT *Fz4^{CKOAP/+}* cells into mutant *Fz4^{AP/-}* cells.

In *Fz4^{CKOAP/+};Sox2-Cre* mice, which produce Cre ubiquitously (Hayashi et al., 2002), the AP reporter is seen throughout the vasculature starting at embryonic day (E)8. Following Cre-mediated recombination in ECs (*Fz4^{CKOAP/+};Tie2-Cre*; Figure 1B; Kisanuki et al., 2001) or in MCs (*Fz4^{CKOAP/+};PDGFRB-Cre*; Foo et al., 2006), *Fz4^{AP}* expression is maintained through adulthood (Figure S1 available online).

In the retina, Fz4 is expressed in ECs, MCs, photoreceptors, and a subset of inner retinal neurons (Figure S1C–S1E; Wang et al., 2001). To determine the cell type(s) responsible for the *Fz4^{-/-}* retinal vascular phenotype, we deleted Fz4 in ECs with *Tie2-Cre*, in MCs with *PDGFRB-Cre* (Cre-mediated recombination in this case is incomplete), and in most or all retinal neurons and glia with *Rx-Cre* (Swindell et al., 2006). In the latter two cases, there was little or no change in retinal vascular morphology (Figure S2), although, as described more fully below, the *Fz4^{AP/-}* MCs in the *Fz4^{CKOAP/-};PDGFRB-Cre* retina are abnormal. By contrast, in adult *Fz4^{CKOAP/-};Tie2-Cre* mice, intraretinal capillaries are completely absent, vessels penetrating from the vitreal surface terminate in ball-like clusters, large vessels are dilated and form arterio-venous anastomoses, and intraocular hemorrhages are common (Figure 1C). These retinal vascular defects closely resemble those seen in *Fz4^{-/-}*, *Lrp5^{-/-}*, and *Ndp^{-/-}* mice (see Figure 3A below; Xu et al., 2004; Luhmann et al., 2005b; Xia et al., 2008), indicating that defective Fz4 signaling in retinal ECs (RECs) recapitulates these loss-of-function phenotypes.

To determine whether Fz4 acts in a cell-autonomous manner, we took advantage of the observation that ~5% of *Fz4^{CKOAP/+}*;

Tie2-Cre and *Fz4^{CKOAP/-};Tie2-Cre* retinas exhibit incomplete Cre-mediated recombination (Figure 1D). In incompletely recombined *Fz4^{CKOAP/+};Tie2-Cre* retinas, *Fz4^{AP/+}* RECs populate relatively contiguous territories encompassing both large and small vessels within all three vascular layers. Each of these contiguous territories is presumably composed of the clonal progeny of one or more REC progenitors that underwent a Cre-mediated recombination event (Figure 1Da).

Several distinctive features characterize *Fz4^{AP/-}* RECs in *Fz4^{CKOAP/-};Tie2-Cre* retinas with incomplete recombination (Figures 1Db–1Dd). First, there was a strong predilection for *Fz4^{AP/-}* RECs to remain confined to vessels at the vitreal surface, implicating a requirement for Fz4 signaling specifically in the development of intraretinal capillaries. Second, in contiguous territories populated by *Fz4^{AP/-}* RECs, ball-like clusters of RECs characteristic of the *Fz4^{-/-}* vasculature were seen instead of intraretinal capillaries. Third, at the boundaries of large clones of *Fz4^{AP/-}* RECs, some *Fz4^{AP/-}* RECs were integrated into the adjacent WT capillary network, suggesting that close proximity between WT and *Fz4^{AP/-}* RECs can partially correct the defects associated with loss of Fz4. Fourth, in regions of retina populated by nonrecombined (i.e., AP-negative) RECs, rare *Fz4^{AP/-}* RECs were observed in all classes of blood vessels, including intraretinal capillaries. These *Fz4^{AP/-}* RECs likely arose from late Cre-mediated recombination events among *Fz4^{CKOAP/-}* RECs that were already part of a capillary network, implying that Fz4 is not absolutely or immediately required to maintain the capillary REC phenotype once that phenotype has been established.

Muller Glia Are the Principle Source of Norrin

To localize the site(s) of *Ndp* expression, we inserted an AP reporter into exon 2 in the *Ndp* 5' UTR (Figure 1E). *Ndp* is X-linked, and histochemical staining for AP in *Ndp^{AP}* males showed homogeneous AP staining throughout the retina at postnatal day (P)4, P7, and in adulthood. To visualize individual cell morphologies, we took advantage of the tissue mosaicism generated by X chromosome inactivation in *Ndp^{AP/+}* females. At P4, P7, and adulthood, AP activity was localized to vertical stripes spanning the full thickness of the retina, indicative of expression in Muller glia (Figure 1F). Flat mounts of *Ndp^{AP/+}* female retinas at P7 show no detectable AP expression in retinal ganglion cells (RGCs) or astrocytes (Figure 1F). Thus, Muller glia produce Norrin, which then activates Fz4 on RECs.

Defects in Visual Function in *Fz4^{-/-}* Mice Caused by Retinal Vascular Insufficiency

Vision loss is a hallmark of FEVR, but it is not known whether this is entirely due to vascular insufficiency or whether there are contributions from the loss of Fz4 signaling in neurons. To characterize the type, severity, and anatomic origin of visual deficits associated with loss of Norrin/Fz4/Lrp signaling, we measured rod- and cone-mediated full-field electroretinographic (ERG) responses in *Fz4^{-/-}*, *Fz4^{CKOAP/-};Rx-Cre*, and *Fz4^{CKOAP/-};Tie2-Cre* mice and in control WT, *Fz4^{CKOAP/+};Rx-Cre*, and *Fz4^{CKOAP/+};Tie2-Cre* mice (Figures 2A–2C), then analyzed each retina by whole-mount AP histochemistry. Photoreceptor (a-wave) and inner retinal (b-wave) ERG responses in the pan-neuronal/pan-glial Fz4 knockout retina (*Fz4^{CKOAP/-};Rx-Cre*) were

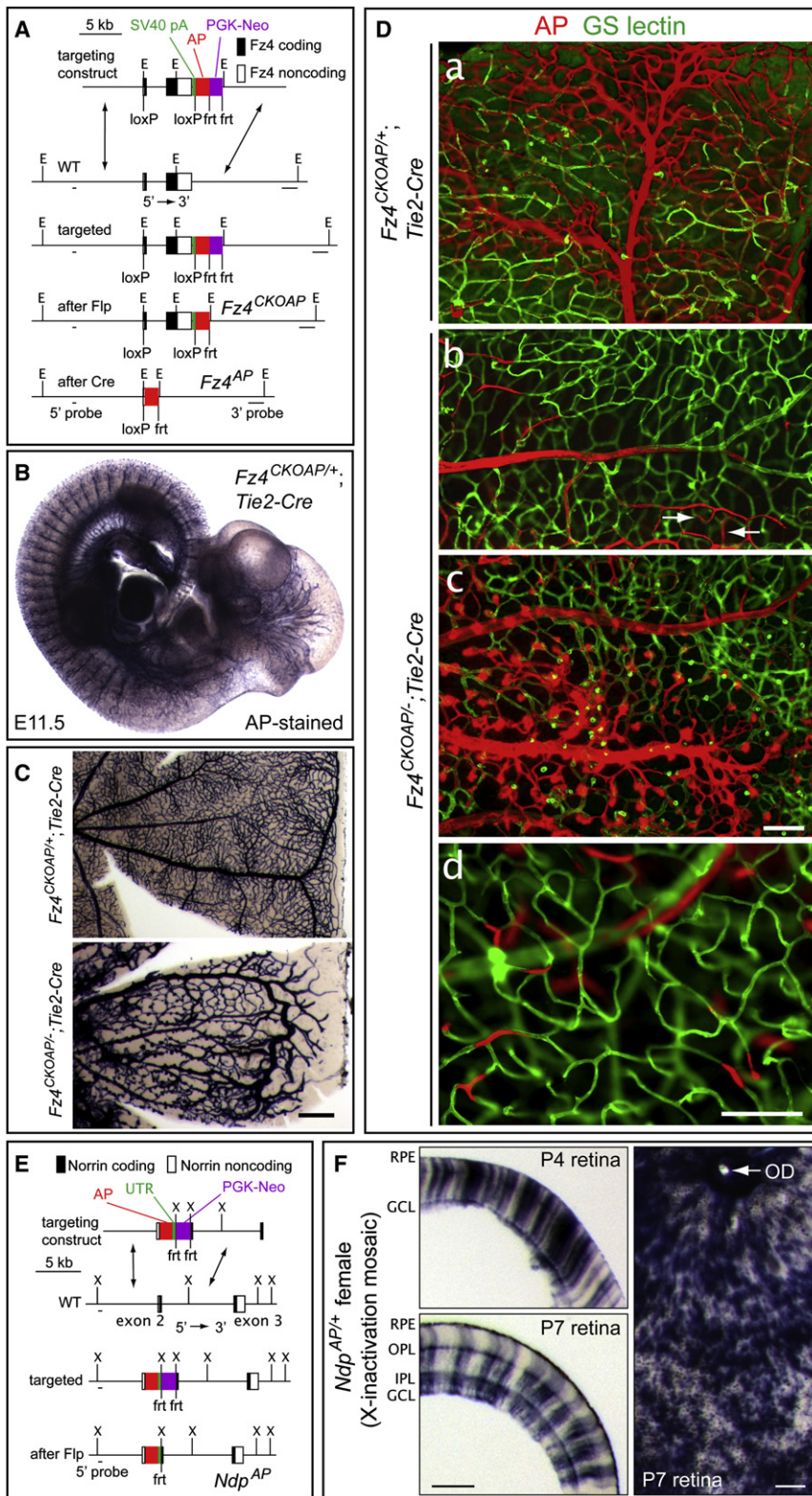


Figure 1. Expression of *Fz4* in Endothelial Cells and *Ndp* in Muller Glia: Absence of *Fz4* Signaling in Endothelial Cells Leads to Severe Defects in Retinal Vascular Development

(A) Structure of the *Fz4*^{CKOAP} allele. *LoxP* sites were placed in the 5' UTR and 3' of the 3' UTR. Cre-mediated recombination deletes *Fz4* coding sequences and activates AP. E, EcoRI.

(B) *Fz4* is expressed in ECs as determined by AP histochemistry of a *Fz4*^{CKOAP/+}; *Tie2-Cre* E11.5 embryo.

(C) AP histochemistry of adult retinas. *Fz4*^{AP/+} ECs produce a WT vasculature, and *Fz4*^{AP/-} ECs produce a vascular phenotype identical to that of *Fz4*^{-/-} mice. Scale bar, 200 μm.

(D) Mosaic analysis of *Fz4*^{CKOAP/+}; *Tie2-Cre* and *Fz4*^{CKOAP/-}; *Tie2-Cre* retinas with incomplete Cre-mediated recombination. (a) *Fz4*^{AP/+} ECs populate large vessels and capillaries. (b-d) Vessels are populated by *Fz4*^{AP/-} ECs only if they reside on the vitreal surface (b and c); intraretinal growth of *Fz4*^{AP/-} ECs typically ends in a compact ball of cells (c), with some *Fz4*^{AP/-} ECs incorporated into the adjacent WT capillary network (white arrows in b). (d) Rare *Tie2-Cre*-mediated recombination events generate isolated *Fz4*^{AP/-} ECs within mature intraretinal capillaries. Scale bar, 100 μm. (E) Structure of the *Ndp*^{AP} allele. UTR, rabbit beta-globin 3' UTR; X, XbaI.

(F) AP-stained retinas from *Ndp*^{AP/+} female mice. 40 μm cross-sections at P4 and P7 (left) and a flat mount at P7 (right). AP+ Muller glia span the full thickness of the retina. The brown dots in the flat mount are adherent RPE cells. Scale bars, 100 μm. RPE, retinal pigment epithelium; OPL, outer plexiform layer; IPL, inner plexiform layer; GCL, ganglion cell layer; OD, optic disc.

retinal ON pathway (McCall and Gregg, 2008). This ERG phenotype matches that reported previously for *Ndp*^{-/-} mice (Luhmann et al., 2005b). Despite the expression of *Fz4* in photoreceptors and other retinal neurons, these observations indicate that the ERG defect is caused by the absence of *Norrin*/*Fz4*/*Lrp* signaling in the vasculature rather than in neurons.

To assess the behavioral consequences of retinal vascular dysfunction, we tested the optokinetic reflex (OKR) and the pupillary light response. OKR testing showed that, in the absence of *Fz4*, rod and cone signals are not transmitted effectively, and this block arises from a primary defect in the vasculature (Figures 2D and 2E). Interestingly, the

indistinguishable from WT. By contrast, in *Fz4*^{-/-} and *Fz4*^{CKOAP/-}; *Tie2-Cre* mice, the a-wave was unaffected, but the b-wave was markedly reduced, indicating a defect in the inner

OKR defect in *Fz4*^{-/-} and *Fz4*^{CKOAP/-}; *Tie2-Cre* mice is more severe than the OKR defect associated with genetic silencing of the ON pathway in *mGluR6*^{-/-} mice (Iwakabe et al., 1997),

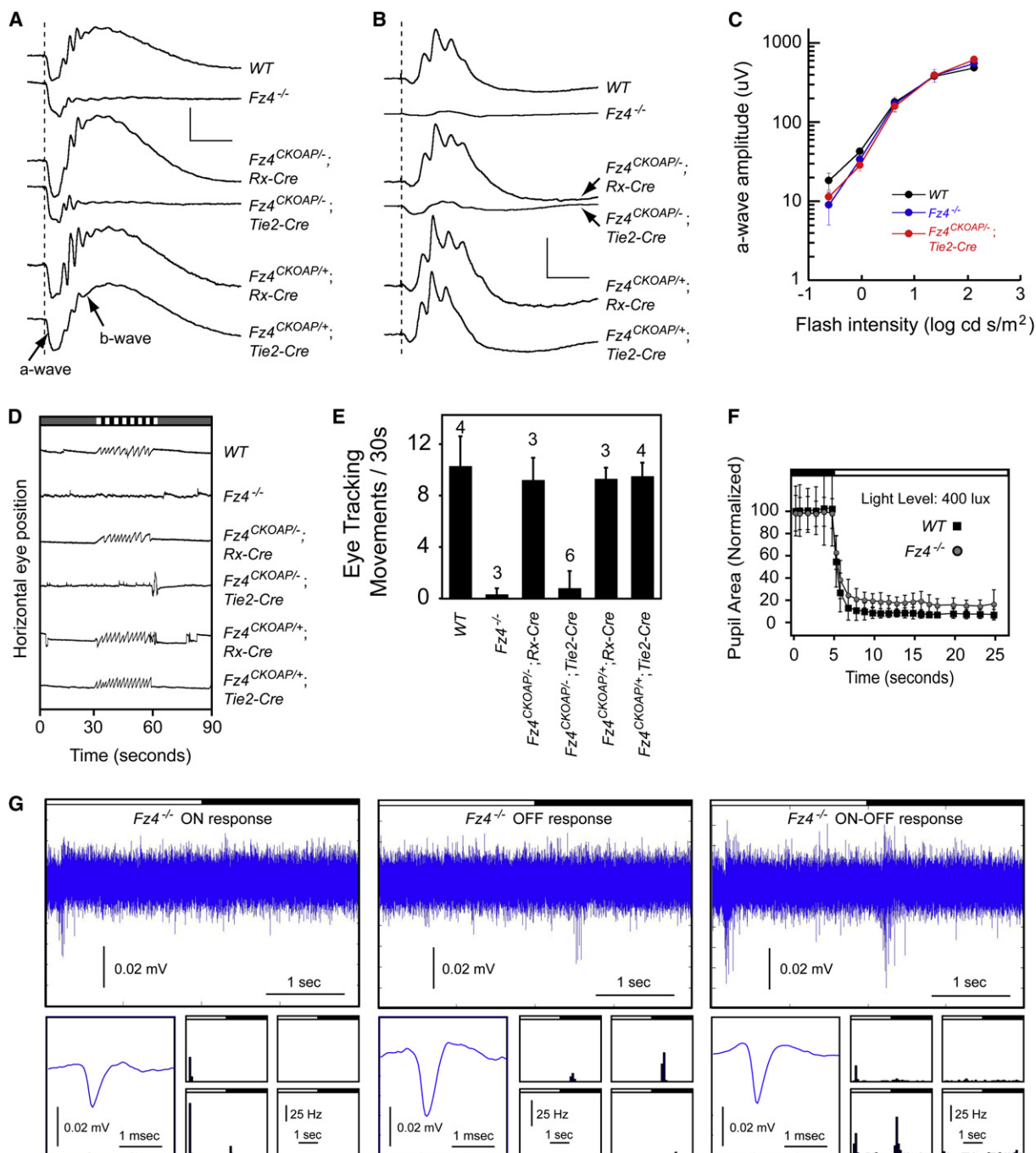


Figure 2. Deficits in Visual Function Associated with Retinal Vascular Insufficiency

(A) Rod ERG responses to a strobe flash (dashed line) presented in darkness. Scale bars, 500 μ m and 50 ms.

(B) Cone ERG responses to a strobe flash (dashed line) superimposed upon a steady rod-desensitizing adapting field. Scale bars, 200 μ m and 50 ms.

(C) Amplitude of the dark-adapted a-wave measured 8 ms after the flash, as a function of stimulus intensity. There is no statistically significant difference between control a-waves (averages of *Fz4*^{+/+}, *Fz4*^{+/-}, *Fz4*^{CKOAP/+}; *Rx-Cre*, and *Fz4*^{CKOAP/+}; *Tie2-Cre*) and *Fz4*^{-/-} and *Fz4*^{CKOAP/-}; *Tie2-Cre* a-waves. The number of mice tested were as follows: *Fz4*^{+/+} (2), *Fz4*^{+/-} (4), *Fz4*^{CKOAP/+}; *Rx-Cre* (6), *Fz4*^{CKOAP/+}; *Tie2-Cre* (5), *Fz4*^{-/-} (7), *Fz4*^{CKOAP/-}; *Rx-Cre* (6), and *Fz4*^{CKOAP/-}; *Tie2-Cre* (6). Bars, standard deviation (SD).

(D) The OKR to horizontally rotating vertical stripes (striped bar at top) is lost in *Fz4*^{-/-} and *Fz4*^{CKOAP/-}; *Tie2-Cre* mice.

(E) Quantification of the OKR. The number of mice tested is indicated. Among six *Fz4*^{CKOAP/-}; *Tie2-Cre* tested, one showed a weak OKR and was subsequently found to exhibit incomplete Cre-mediated recombination in the retinal vasculature; the remaining five mice showed no OKR. Bars, SD.

(F) *Fz4*^{-/-} and WT littermates show nearly normal pupil constriction following the onset of a 400 lux stimulus (open bar at top). For each genotype, four mice were tested six times and the 24 responses averaged. Bars, SD.

consistent with a model in which the OKR is driven by both the ON and OFF pathways and indicating that both pathways are compromised in $Fz4^{-/-}$ and $Fz4^{CKOAP/-};Tie2-Cre$ mice.

In contrast to the OKR, light-dependent pupil constriction is minimally impaired by the loss of $Fz4$ (Figure 2F), indicating that the melanopsin-expressing intrinsically photosensitive RGCs, which control pupil constriction (Hattar et al., 2003), remain active in the $Fz4^{-/-}$ retina. These data are consistent with earlier studies showing an absent OKR and nearly normal pupil response in mice that are missing both rod and cone function (Hattar et al., 2003; Cahill and Nathans, 2008).

Despite a chronic vascular insufficiency that precludes normal responses of horizontal, bipolar, and/or amacrine cells in Ndp^{-} , $Fz4^{-/-}$, and $Fz4^{CKOAP/-};Tie2-Cre$ retinas, the mutant retinas appear surprisingly normal (Figures 3A, S3, and S4). This observation suggested that it might be possible to revive these neurons to the point where they could transmit photoreceptor signals. To test this idea, we recorded light responses from the ganglion cell layer in WT and $Fz4^{-/-}$ retinas ex vivo using a multi-electrode array (Meister et al., 1994). In this preparation, oxygen, glucose, and other small molecules passively diffuse into the retina, obviating any need for the vasculature. As seen in Figure 2G, ganglion cell layer neurons in the adult $Fz4^{-/-}$ retina exhibit ON, OFF, and ON-OFF action potential responses to local changes in illumination. The number of ON, OFF, and ON-OFF subtypes identified in the retinas of $Fz4^{-/-}$ mice were 4, 55, and 12, respectively; the number of ON, OFF, and ON-OFF subtypes identified in the retinas of WT littermates were 19, 21, and 16, respectively. In sum, the ERG, OKR, pupil constriction, and multi-electrode array experiments indicate that a chronic absence of intraretinal capillaries leads to a severe and localized—but still largely reversible—block to neurotransmission through the inner nuclear layer.

Norrin/Fz4/Lrp Signaling Controls Vascular Growth and EC-MC Interactions

To assess the relative contributions of Norrin, Fz4, and Lrp5 to retinal vascular growth and patterning, we compared WT, $Fz4^{-/-}$, $Fz4^{CKOAP/-};Tie2-Cre$, $Lrp5^{-/-}$, and Ndp^{-} mice with respect to (1) the appearance of the vasculature in the adult (Figure 3A), (2) vascular growth during the first postnatal week (Figures 3B and 3C), and (3) vascular density at P7 (Figure 3D). Although the initial vascular invasion of the retina was unaffected by any of the mutations, $Fz4^{-/-}$, $Fz4^{CKOAP/-};Tie2-Cre$, and Ndp^{-} retinas exhibited retarded vascular growth and sparse vascular coverage, as reported previously in $Fz4^{-/-}$ and Ndp^{-} retinas (Xu et al., 2004; Luhmann et al., 2005b). By each of these measures, $Lrp5^{-/-}$ retinas show a more modest phenotype. These observations suggest that Norrin is the only Fz4 ligand relevant to retinal angiogenesis, and that there may be a partial contribution to coreceptor function from Lrp6, a close homolog of Lrp5.

MCs are required both to stabilize the nascent vasculature and to maintain the permeability barrier of the mature vasculature and, in particular, the blood brain barrier (BBB) and the blood retina barrier (BRB) (Armulik et al., 2005). The sparse vasculature in $Fz4^{-/-}$ and Ndp^{-} retinas (Figures 3B and 3D), the defective cerebellar BBB associated with loss of $Fz4$ (see Figure 4A below), and the defective BRB associated with loss of Ndp or $Fz4$ (Xu et al., 2004; Luhmann et al., 2005b) suggested that Norrin/Fz4/Lrp signaling might control EC-MC interactions. This hypothesis is consistent with the expression of $Fz4$ in both ECs and MCs, and, as described below, it is strongly supported by the observation of defective EC-MC interactions in the absence of Norrin/Fz4/Lrp signaling.

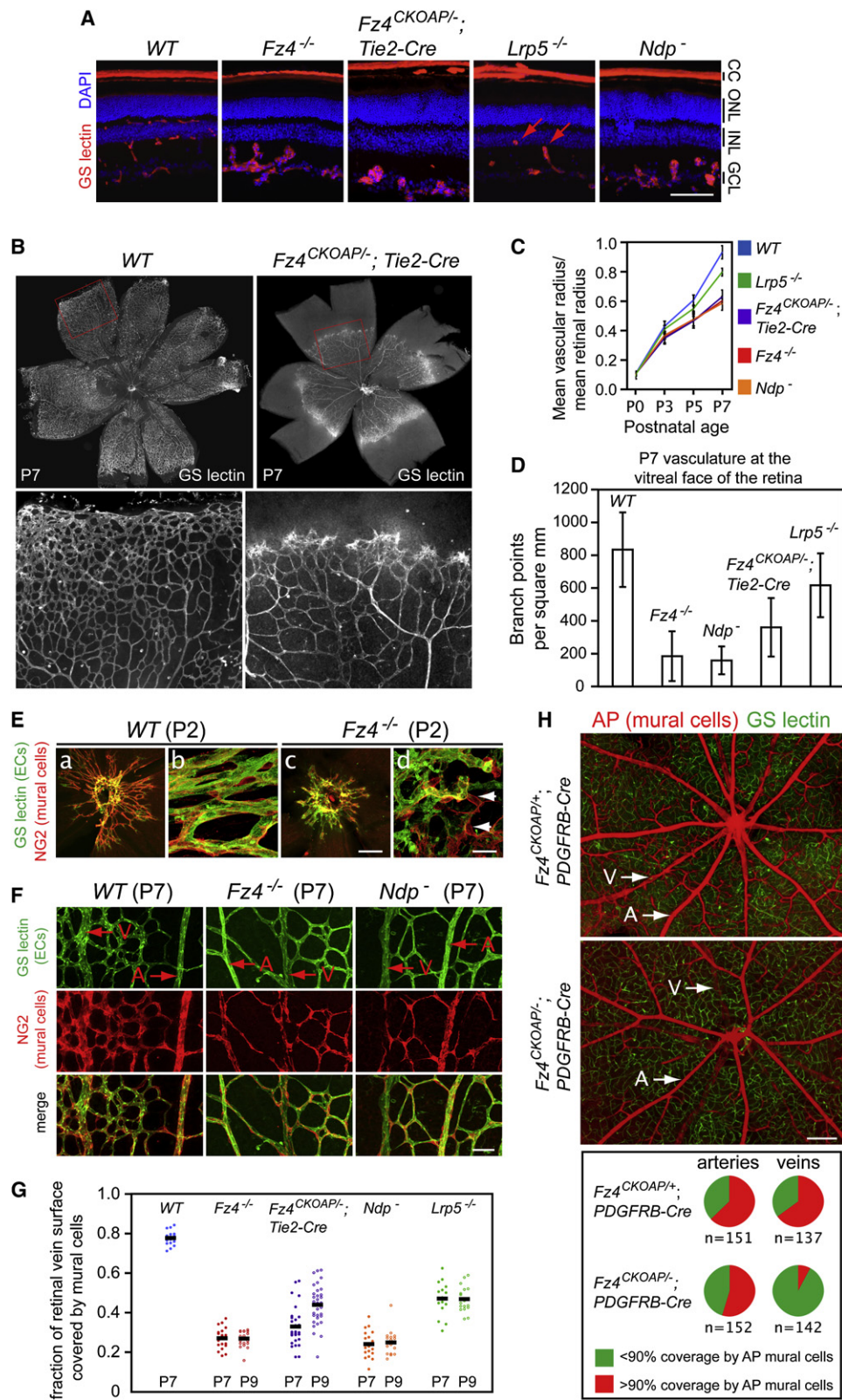
At P2, at the start of REC outgrowth, EC-MC interactions are already strikingly aberrant in $Fz4^{-/-}$ retinas, indicating an initial failure of EC-MC recognition or adhesion (Figure 3E). At P7, in both $Fz4^{-/-}$ and Ndp^{-} retinas, MCs are thinner and MC density is lower than in WT retinas, resulting in incomplete coverage of veins and capillaries (Figure 3F). In contrast, arterial coverage by vascular smooth muscle cells (vSMCs) is minimally affected in $Fz4^{-/-}$ and $Norrin^{-}$ retinas. Quantification of MC coverage of the radial veins at P7 shows a defect in $Fz4^{CKOAP/-};Tie2-Cre$ retinas that is almost as severe as that found in $Fz4^{-/-}$ and Ndp^{-} retinas; a milder defect is found in $Lrp5^{-/-}$ retinas (Figure 3G). Interestingly, between P7 and P9, MC coverage of veins in $Fz4^{CKOAP/-};Tie2-Cre$ retinas increased significantly ($p < 0.0001$, Student's *t* test), and by P9 it was higher than that in $Fz4^{-/-}$ or $Norrin^{-}$ retinas ($p < 0.0001$, ANOVA).

The difference in the severity of MC coverage defects between $Fz4^{CKOAP/-};Tie2-Cre$ and $Fz4^{-/-}$ retinas (Figure 3G) indicates that EC-MC interactions depend on Fz4 function in at least one cell type in addition to ECs. To test the obvious possibility that the additional cell type is the MC, we visualized $Fz4^{AP/+}$ MCs in $Fz4^{CKOAP/+};PDGFRB-Cre$ retinas and $Fz4^{AP/-}$ MCs in $Fz4^{CKOAP/-};PDGFRB-Cre$ retinas (Figure 3H). Whereas $Fz4^{AP/+}$ MCs efficiently cover both arteries and veins, $Fz4^{AP/-}$ MCs provide substantially lower coverage of veins: in control $Fz4^{CKOAP/+};PDGFRB-Cre$ retinas at P30, 65% of veins and 63% of arteries show >90% coverage by AP-expressing MCs, whereas in $Fz4^{CKOAP/-};PDGFRB-Cre$ retinas at P30, 55% of arteries and only 8% of veins show >90% coverage. These results demonstrate an essential role for Norrin/Fz4/Lrp signaling in ECs to promote vascular growth, and in both ECs and MCs to promote their mutual interactions.

Fz4 Is Required for Vascular Integrity in the Cerebellum

In addition to retinal vascular defects, $Fz4^{-/-}$ mice exhibit a progressive cerebellar degeneration (Wang et al., 2001). Although Ndp^{-} mice do not exhibit this phenotype, both $Fz4^{-/-}$ and Ndp^{-} mice show a progressive reduction in vascular density in the cerebellum, suggesting a defect in vascular integrity and/or stability (Xu et al., 2004; Luhmann et al., 2008). Consistent with

(G) Extracellular multi-electrode array recordings of light responses from ganglion cell layer neurons from 2-month-old $Fz4^{-/-}$ retinas. Upper panels, individual ON, OFF, and ON-OFF cell responses to 2 seconds of light followed by 2 seconds of darkness (black and white bars). Shown for each cell is the averaged spike waveform obtained from ten repetitions of the alternating light/dark stimulus (lower left panels) and a peri-stimulus histogram showing the frequency (Hz) and timing of spikes in response to ten repetitions of the stimulus at each of four stimulus locations comprising a 2x2 subset of the 5x5 stimulus array (lower panels; 100 ms bins).



this hypothesis, we commonly observe hemorrhages in the cerebellum but not in the cerebral cortex in $Fz4^{-/-}$ brains.

To test the role of Fz4 signaling in the cerebellar vasculature, we monitored the BBB by immunostaining for mouse IgG in freshly frozen sections of $Fz4^{CKOAP/-};Tie2-Cre$, control $Fz4^{CKOAP/+};Tie2-Cre$, and Ndp^{-} brains. In the absence of an intact BBB, IgG can pass from the intravascular space into the surrounding tissues. As seen in Figure 4A, in brain sections that include both cerebellum and cortex, only the $Fz4^{CKOAP/-};Tie2-Cre$ cerebellum showed a defect in BBB integrity. An identical vascular phenotype was seen in $Fz4^{-/-}$ brains (data not shown), and transmission electron microscopy of WT and $Fz4^{-/-}$ cerebella shows, in the latter, misshapen capillaries and protrusions of endothelial processes into the vessel lumen (Figure S5). Importantly, quantification of granule cell apoptosis in WT, $Fz4^{CKOAP/-};Tie2-Cre$, and $Fz4^{-/-}$ cerebella at P18 demonstrate that the $Fz4^{-/-}$ cerebellar degeneration is of vascular origin (Figures 4B and 4C).

These experiments indicate a major role for Fz4 signaling in maintaining the integrity of the cerebellar vasculature, presumably in response to Norrin and/or Wnt binding. Consistent with this conclusion, recent evidence indicates that Wnt7a and Wnt7b expressed in the developing central nervous system (CNS) promote CNS-type vascular development, including BBB development (Stenman et al., 2008; Daneman et al., 2009).

Overactivation of Fz4/Lrp Signaling Disrupts Embryonic Angiogenesis

A growing number of observations imply that Norrin/Fz4 or Wnt/Fz4 signaling plays a role in vascular development or maintenance outside of the retina. First, $Fz4^{-/-}$ and Ndp^{-} mice exhibit a progressive loss of blood vessels within the stria vascularis that is associated with hearing loss (Rehm et al., 2002; Xu et al., 2004); similarly, more than a third of Norrie disease patients experience hearing loss (Berger and Ropers, 2001). Second, in two families, *NDP* mutations have been associated with both Norrie disease and peripheral vascular insufficiency (Rehm et al., 1997; Michaelides et al., 2004). Third, female $Fz4^{-/-}$ and Ndp^{-} mice exhibit defects in corpora lutea formation and endo-

metrial vascularization of the placenta, respectively, processes that require rapid vascular growth and remodeling (Hsieh et al., 2005; Luhmann et al., 2005a). The observation of $Fz4^{AP}$ expression throughout the developing and adult vasculature (Figures 1B and S1) is also consistent with a wider role for Fz4 signaling in vascular biology.

To explore this idea, we asked whether Fz4 signaling induced by ectopic Norrin could modify vascular development. To produce Norrin ectopically, we targeted a Cre-activated *Norrin* cassette to the *Ubiquitin-b (Ubb)* locus, the site of transgene insertion in the *Z/AP* mouse line (Figure 5A; Lobe et al., 1999; T. Rotolo and J.N., unpublished data); we will refer to this knockin allele as “*Z/Norrin*.” In *Z/Norrin;Sox2-Cre* mice, Cre-mediated recombination occurs early in embryogenesis, producing a severe disruption in angiogenesis and midgestational lethality. Although most *Z/Norrin;Sox2-Cre* embryos are still alive at E10.5, they are growth retarded and have severely disorganized embryo and yolk sac vasculatures (Figures 5B and 5C). Moreover, at E9.5, when vSMCs begin to surround the dorsal aorta in WT embryos, the aorta in *Z/Norrin;Sox2-Cre* embryos has no associated vSMCs (Figure 5D).

The defects caused by Norrin overproduction are fully suppressed in a $Fz4^{-/-}$ background (Figure 5E). Interestingly, in a $Fz4^{+/-}$ background, the *Z/Norrin;Sox2-Cre* phenotype is partially suppressed, leading to a milder phenotype at E10.5 and occasional survivors at E18 (Figures 5F and 5I). These experiments indicate that, in the embryonic vasculature, Fz4 is the major receptor for Norrin, and Fz4 signaling can potentially modulate angiogenesis. The *Fz4* dosage sensitivity is consistent with the mild-to-moderate vascular insufficiency observed in humans with FEVR who are heterozygous for presumptive loss-of-function or hypomorphic mutations in *Fz4*; a more severe phenotype has been reported in one individual with homozygous mutation of *Fz4* (Robitaille et al., 2002; Toomes et al., 2004; Xu et al., 2004; Qin et al., 2005; Kondo et al., 2007).

Partial rescue of the Norrin overproduction phenotype was also obtained in an $Lrp5^{-/-}$ background (Figures 5G and 5H). The failure of the $Lrp5^{-/-}$ background to fully rescue the Norrin overproduction phenotype could reflect signaling via *Lrp6*,

Figure 3. Fz4 Signaling Is Required for Retinal Vascular Growth and for Normal EC-MC Interactions

- (A) WT adult retina shows the normal two tiers of capillaries. $Fz4^{-/-}$, $Fz4^{CKOAP/-};Tie2-Cre$, and Ndp^{-} retinas fail to develop intraretinal capillaries; $Lrp5^{-/-}$ retinas show partial development of the inner tier (red arrows). CC, choriocapillaris; ONL, outer nuclear layer; INL, inner nuclear layer; GCL, ganglion cell layer. Scale bar, 100 μ m.
- (B) Delayed EC migration and impoverished vascular network formation in $Fz4^{CKOAP/-};Tie2-Cre$ retinas at P7. Regions boxed in red are shown at higher magnification; the vessels reside on the vitreal surface. Scale bar, 20 μ m.
- (C) Quantification of EC spreading from the optic disc. $Fz4^{-/-}$, $Fz4^{CKOAP/-};Tie2-Cre$, and Ndp^{-} retinas are equivalently defective; $Lrp5^{-/-}$ retinas show a milder defect. For each genotype, 6–10 retinas were analyzed per time point. Bars, standard error of the mean (SEM).
- (D) Quantification of vascular density at the vitreal surface. $Lrp5^{-/-}$ retinas show a mild defect, $Fz4^{CKOAP/-};Tie2-Cre$ retinas show an intermediate defect, and $Fz4^{-/-}$ and Ndp^{-} retinas show equivalently severe defects. Bars, SEM.
- (E) P2 retina flat mounts show that MC coverage of ECs in $Fz4^{-/-}$ retinas is aberrant from the earliest stages of vascular development (white arrows, detached MCs). (a and c) Images centered at the optic disc; scale bar, 200 μ m. (b and d) Scale bar, 20 μ m.
- (F) P7 retina flat mounts show decreased MC coverage of capillaries and veins (V), with minimal effect on arteries (A), in $Fz4^{-/-}$ and Ndp^{-} retinas. Scale bar, 50 μ m.
- (G) Quantification of the surface area of the principal retinal veins covered by MCs. Mutant retinas were examined at P7 and P9 because vascular migration is delayed ~2 days (Figures 3B and 3C). Each data point represents a different vein. Black bars, averages. P7 versus P9 $Fz4^{CKOAP/-};Tie2-Cre$ ($p < 0.0001$, Student's t test); P9 $Fz4^{CKOAP/-};Tie2-Cre$ versus $Fz4^{-/-}$ or *Norrin*⁻ ($p < 0.0001$, ANOVA). Each mutant at either P7 or P9 versus WT at P7 ($p < 0.0001$, Student's t test).
- (H) $Fz4^{AP/+}$ and $Fz4^{AP/-}$ MCs visualized at P30 in the $Fz4^{CKOAP/+};PDGFRB-Cre$ retina (upper panel) and $Fz4^{CKOAP/-};PDGFRB-Cre$ retina (lower panel), respectively. GS-lectin staining shows normal capillary architecture in both retinas. Recombination is more efficient in MCs on large vessels compared to capillaries. Pie charts show MC coverage at P30, with the number of scored veins (V) and arteries (A) indicated. The artery coverage difference is not statistically significant ($p = 0.162$); for the vein coverage, $p = 10^{-25}$ (Fisher's exact test). Scale bar, 200 μ m.

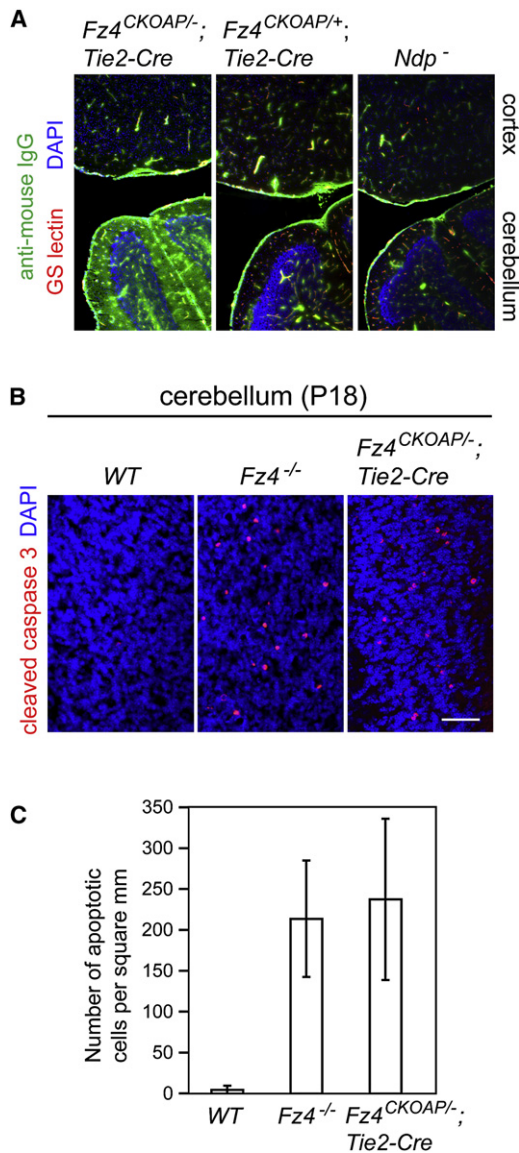


Figure 4. Vascular Basis of Cerebellar Degeneration in *Fz4^{-/-}* Mice
(A) Loss of vascular integrity occurs in the *Fz4^{CKOAP/-}; Tie2-Cre* cerebellum but not cerebral cortex as determined by staining for IgG in freshly frozen tissue. Scale bar, 300 μ m.

(B and C) Large numbers of apoptotic granule cells in the P18 cerebellum are observed with deletion of *Fz4* in ECs. $p < 10^{-6}$ for *Fz4^{CKOAP/-}; Tie2-Cre* versus WT and for *Fz4^{-/-}* versus WT (Student's *t* test). Scale bar in (B), 50 μ m. Bars, SEM.

which is expressed in both vascular and nonvascular cells in the E10.5 embryo and yolk sac (Figure S6).

Defects in the Production of Capillary-like Structures by *Fz4^{-/-}* REC Lines

In all of the experiments described above, Norrin/Fz4/Lrp signaling was studied in vivo. To reduce this system to its simplest components, we asked whether RECs in culture recapitulate any of the phenotypic differences between WT and

mutant mouse lines. To isolate the requisite REC lines, we crossed *Fz4^{-/-}*, *Ndp^{-/-}*, and *Lrp5^{-/-}* alleles into the "Immorto-mouse" line (Jat et al., 1991). To prepare REC lines, adult retinas were dissociated, and vascular fragments were immuno-affinity purified using an anti-PECAM mAb (Figure 6A; Matsubara et al., 2000; Su et al., 2003). REC lines—defined as VE-cadherin⁺, PECAM⁺, and NG2⁻ epithelial cells—were obtained from WT (4 lines), *Ndp^{-/-}* (7 lines), *Lrp5^{-/-}* (3 lines), and *Fz4^{-/-}* (6 lines) Immorto-mice (Figure 6A).

In a simple test of cell migration, WT, *Fz4^{-/-}*, and *Lrp5^{-/-}* REC lines showed substantial motility over a 24 hr interval in response to scratch wounding of a confluent monolayer (Figure 6B). An assay that more closely resembles vascular development is the formation of capillary-like networks by RECs cultured on Matrigel. In this assay, 4/4 WT, 3/3 *Lrp5^{-/-}*, and 6/7 *Ndp^{-/-}* REC lines showed robust network formation, whereas 6/6 *Fz4^{-/-}* REC lines showed virtually no changes by the end of the standard 6 hour incubation (Figure 6C). Interestingly, when WT and *Fz4^{-/-}* RECs were plated together on Matrigel, many of the *Fz4^{-/-}* RECs adopted a more differentiated morphology and were incorporated into the WT capillary-like network (Figure 6D and Movie S1). This cooperative behavior is reminiscent of the in vivo integration of some *Fz4^{AP/-}* RECs into normal-appearing capillaries when mutant and WT RECs are in close proximity (Figure 1D). These observations suggest that *Fz4^{-/-}* RECs are competent to receive, but are unable to send, a cell adhesion or cell motility signal.

It is interesting that *Lrp5^{-/-}* RECs show no apparent defect in the Matrigel assay. Given the intermediate phenotype exhibited by *Lrp5^{-/-}* RECs in vivo (Figures 3A, 3C, 3D, and 3G), we interpret this finding to mean that residual Norrin/Fz4/Lrp signaling—perhaps via Lrp6, which is expressed in RECs at levels comparable to Lrp5 (Figure S6)—may be sufficient to support capillary-like network formation by *Lrp5^{-/-}* RECs.

A Transcriptional Program Controlled by Norrin/Fz4/Lrp Signaling

To explore the underlying molecular effectors of the angiogenic response to Norrin/Fz4/Lrp signaling, we analyzed genome-wide changes in transcript abundance resulting from either increased or decreased signaling. To identify the primary response to increased Norrin/Fz4/Lrp signaling, we compared WT and *Z/Norrin; Sox2-Cre* yolk sacs at E8.5 (prior to the appearance of any obvious Norrin-induced vascular changes). The yolk sac was chosen because it is easily dissected and it consists only of epithelial and vascular cells. Sixty-six transcripts exhibited a >2-fold change with a *p* value <0.05, and many code for proteins involved in cell-cell signaling and extracellular matrix (ECM) structure and/or remodeling (Figure 7 and Table S1). By E10.5, when vascular morphology in the *Z/Norrin; Sox2-Cre* yolk sac is highly aberrant (Figures 5B and 5C), the number of transcripts with a >2-fold change had increased to 400. Interestingly, the transcript coding for Sox17—an HMG box transcription factor that has been implicated in endothelial development in mice and zebrafish (Matsui et al., 2006; Sakamoto et al., 2007; Pendeville et al., 2008)—was among the several transcripts with the highest increases in abundance in response to Norrin overexpression (Figure 7A).

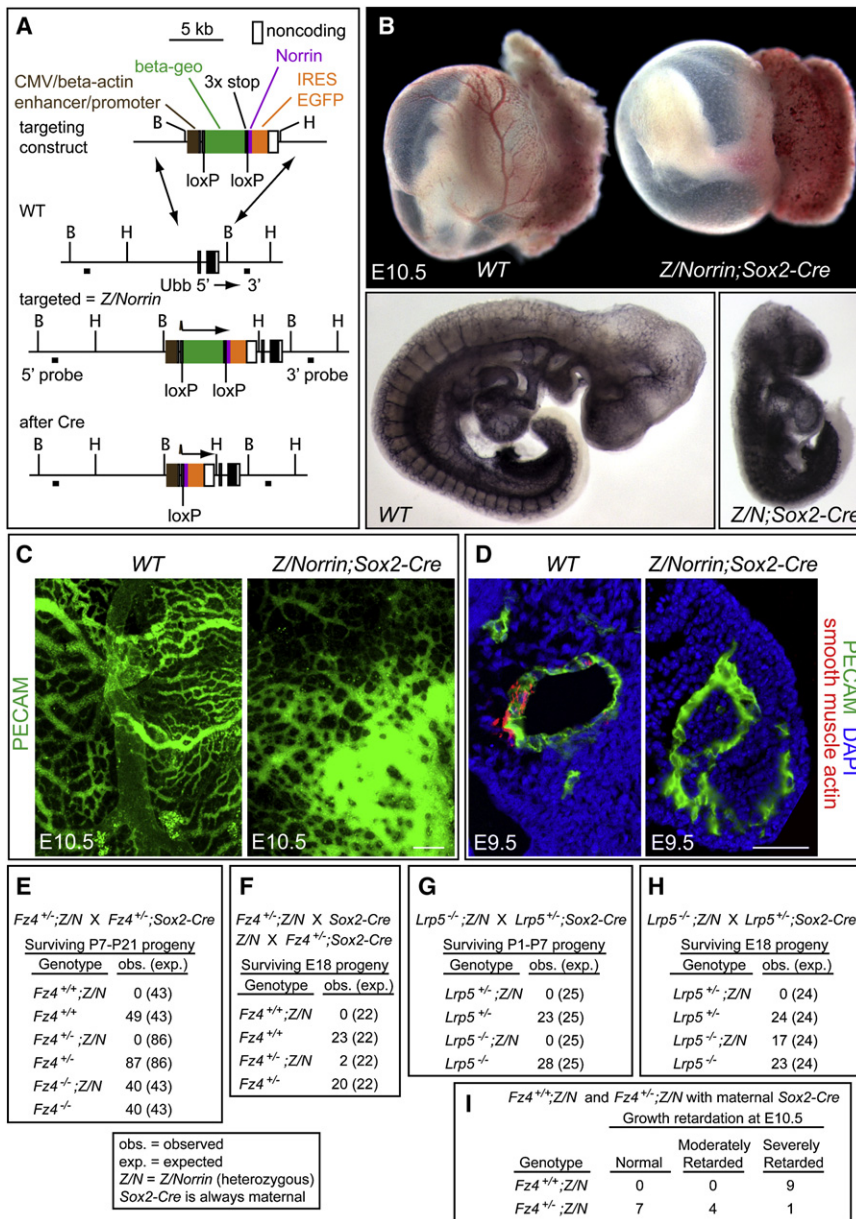


Figure 5. Ubiquitous Production of Norrin during Embryogenesis Leads to Vascular Disorganization

(A) Targeted insertion of a Cre-activated Norrin expression cassette upstream of the *Ubb* gene. Cre-mediated recombination eliminates a *loxP*-flanked *beta-geo* and three transcription termination sites, permitting transcription of *Norrin-IRES-EGFP*.

(B) Upper panel, *Z/Norrin;Sox2-Cre* embryos have a severe defect in yolk sac vascular development and retarded embryonic growth at E10.5. Lower panels, anti-PECAM immunostaining at E10.5 shows generalized vascular disorganization in a *Z/Norrin;Sox2-Cre* embryo (right).

(C) Flat-mounted E10.5 yolk sacs show an orderly hierarchy of vessel sizes in WT (left) and undeveloped and disorganized vasculature in a *Z/Norrin;Sox2-Cre* yolk sac (right). Scale bar, 200 μ m.

(D) Dorsal aorta at E9.5. vSMCs are absent in the *Z/Norrin;Sox2-Cre* aorta. Scale bar, 50 μ m.

(E–I) Midgestational lethality caused by ubiquitous Norrin production is suppressed in a *Fz4*^{-/-} background (E) and partially suppressed in *Fz4*^{+/-} and *Lrp5*^{-/-} backgrounds (F–I).

immunoaffinity-purified adult WT, *Fz4*^{-/-}, *Lrp5*^{-/-}, and *Ndp*^{-/-} retinal vascular cells and from P16 WT and *Fz4*^{-/-} cerebellar vascular cells (Figure S7 and Table S2). Consistent with the high degree of similarity in their retinal vascular phenotypes, the alterations in *Fz4*^{-/-}, *Lrp5*^{-/-}, and *Ndp*^{-/-} retinal vascular transcriptomes were highly correlated and showed numerous differences versus WT (Figure S7).

The differing behaviors of WT and *Fz4*^{-/-} RECs on Matrigel imply corresponding molecular differences. Indeed, microarray hybridization shows substantial differences between WT and *Fz4*^{-/-} RECs, which presumably reflect *Fz4* signaling in WT but not *Fz4*^{-/-} RECs, perhaps in response to Norrin and/or

To validate these results, the abundance changes of a subset of transcripts were determined by blot hybridization (Figure 7A, right). Increases were observed for transcripts coding for Timp3, a matrix metalloproteinase (MMP) inhibitor, Flt1, which functions as a VEGF sink, and several procollagens and procollagen processing enzymes, whereas a decrease was observed for transcripts encoding plasminogen. Transcripts coding for Flk1, the major mediator of VEGF signaling, remained unchanged. Increased production of vascular basement membrane components and reduced MMP activity and VEGF signaling should stabilize the vasculature and block remodeling.

To characterize the effect on the transcriptome of a decrement in Norrin/*Fz4*/*Lrp* signaling, microarray hybridization was performed with RNA from acutely dissociated and anti-PECAM

Wnts in the fetal bovine serum present in the culture medium (Figure 7B). To directly test the Norrin response of WT and *Fz4*^{-/-} RECs in culture, we cocultured these cells either with a human embryonic kidney 293 cell line that stably expresses Norrin or with control 293 cells, then analyzed their transcripts by microarray hybridization (Figure 7C and Table S4). Earlier experiments with a reporter cell line had shown that coculture with Norrin-secreting 293 cells is an effective way to activate *Fz4*/*Lrp* signaling (Xu et al., 2004). Figure 7C shows that WT and *Fz4*^{-/-} RECs differ markedly in their response to coculture with Norrin-expressing 293 cells. Consistent with the WT versus *Z/Norrin;Sox2-Cre* yolk sac comparisons described above, *Sox17* transcripts are more abundant in WT RECs compared to *Fz4*^{-/-} RECs and are induced by Norrin in WT but not in *Fz4*^{-/-} RECs.

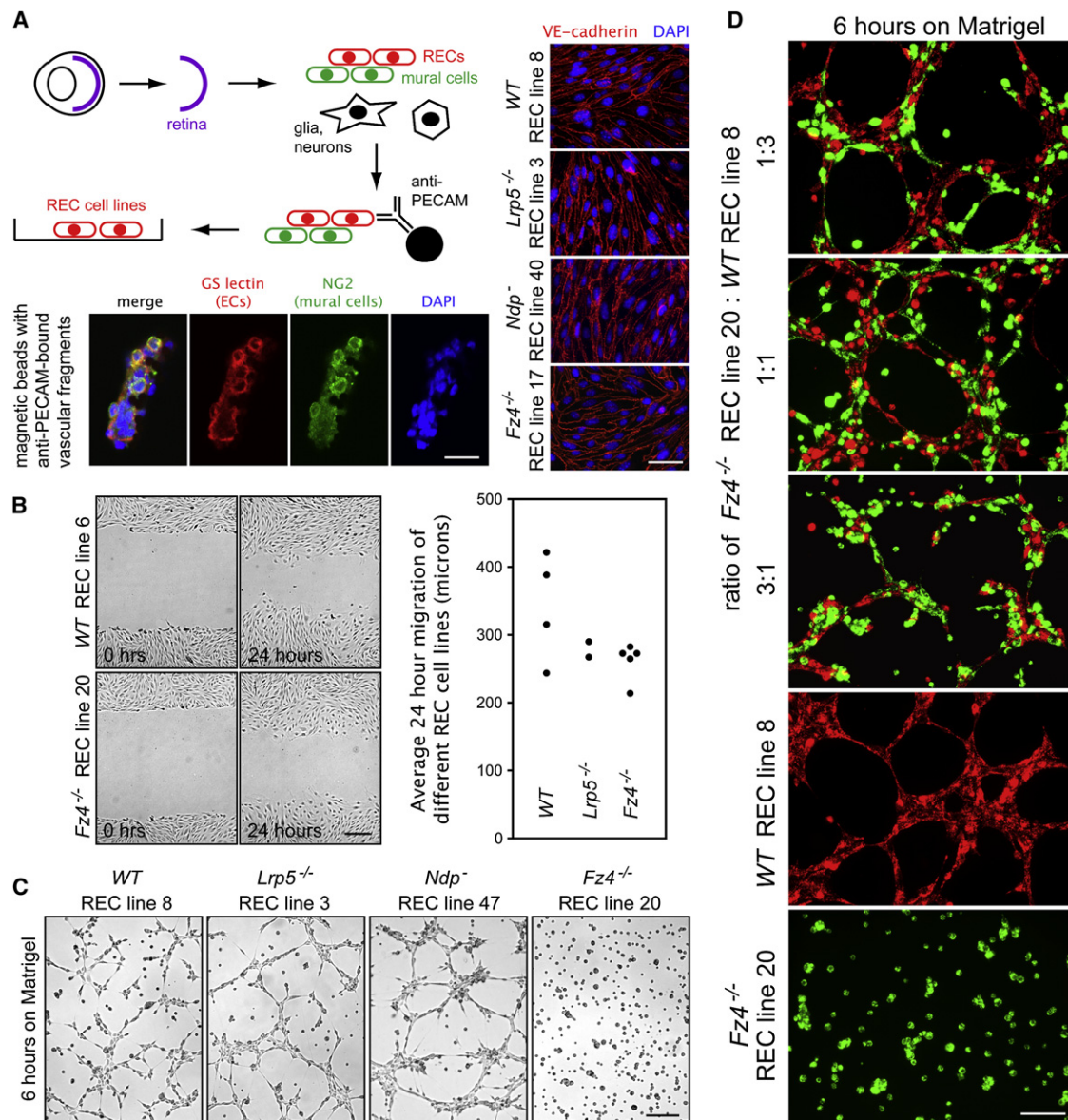


Figure 6. *Fz4*^{-/-} REC Lines Exhibit Defects in the Formation of Capillary-like Structures

(A) Diagram of immunoaffinity purification of vascular fragments (upper) and immunohistochemical characterization of isolated fragments (lower; scale bar 20 μ m) and cloned REC lines (right; scale bar, 50 μ m).

(B) Scratch-induced motility on gelatin-coated dishes. Left, phase-contrast images zero and 24 hr after the scratch; right, quantification of cell motility. Scale bar, 200 μ m.

(C) WT, *Lrp5*^{-/-}, and *Ndp*^{-/-} REC lines form capillary-like structures 6 hr after plating on Matrigel; *Fz4*^{-/-} REC lines do not. Scale bar, 200 μ m.

(D) Matrigel cultures of dil-labeled WT and diO-labeled *Fz4*^{-/-} RECs, mixed immediately before plating at the indicated ratios. Bottom, Matrigel cultures of the pure RECs. Scale bar, 100 μ m.

The various transcriptome comparisons described above reflect Norrin/Fz4/Lrp signaling in different contexts: the yolk sac contains actively proliferating and differentiating embryonic vasculature; the WT or mutant adult retinal vasculature is enriched in or depleted of capillaries, respectively; and REC culture experiments reveal responses ex vivo. It is noteworthy that the two transcripts with abundance changes that correlate most consistently ($p < 0.01$) with increased Fz4/Lrp signaling

code for SparcL1/Hevin/SC1/MAST9, an ECM-associated protein implicated in vascular remodeling (Girard and Springer, 1996; Barker et al., 2005), and Sox17 (Figure 7D).

Sox17 Mediates Norrin/Fz4/Lrp-Dependent Production of Capillary-like Structures

Our observation that the abundance of Sox17 transcripts is increased by Norrin/Fz4/Lrp signaling in a variety of contexts

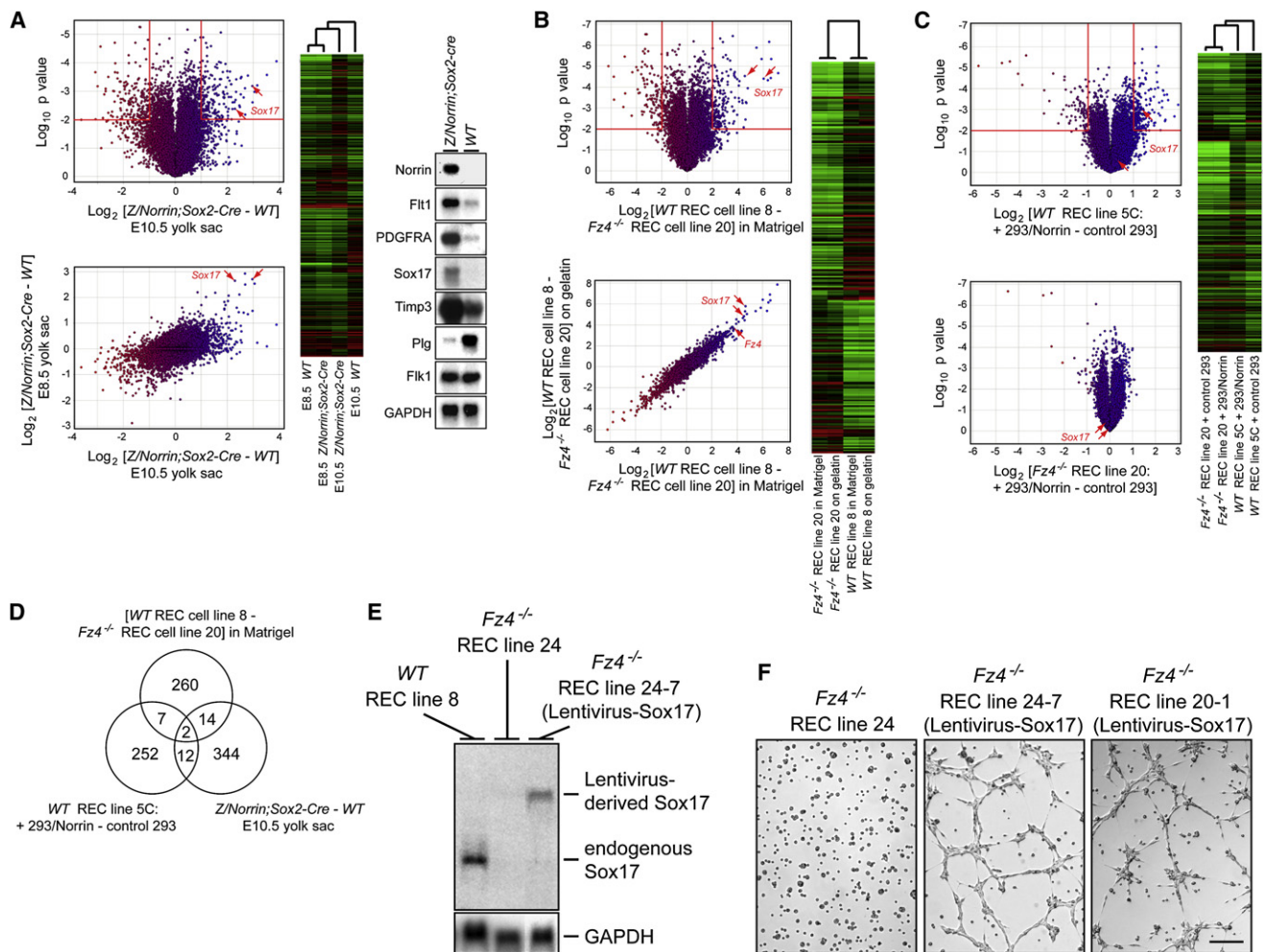


Figure 7. Sox17 Plays a Critical Role in the Norrin/Fz4/Lrp-Dependent Transcriptional Program in Endothelial Cells

(A) Norrin-induced changes in transcript abundances in WT versus Z/Norrin;Sox2-Cre yolk sacs at E8.5 and E10.5. The heat map shows differences greater than 2-fold with a p value <0.01 in the E10.5 WT versus Z/Norrin;Sox2-Cre yolk sac comparison (red borders in upper plot). RNA blot hybridization of selected transcripts in E10.5 WT and Z/Norrin;Sox2-Cre yolk sacs confirms increased abundances of *Norrin*, *Flt1*, *PDGFRA*, *Sox17*, and *Timp3* transcripts, decreased abundances of *Plasminogen* (*Plg*) transcripts, and unaltered abundance of *Flk1* and *GAPDH* transcripts. Microarray data in this figure are averages of three independent experiments.

(B) Transcript differences between WT and Fz4^{-/-} REC lines (upper plot) are largely unaffected by growth on Matrigel versus gelatin (lower plot). The heat map shows differences greater than 4-fold with a p value <0.01 in WT versus Fz4^{-/-} REC lines (red borders in upper plot).

(C) Norrin-induced changes in transcript abundance in WT (upper plot) but not Fz4^{-/-} (lower plot) REC lines. The heat map shows differences greater than 2-fold with a p value <0.01 when WT RECs were cocultured with 293/Norrin versus control 293 cells (red borders in upper plot).

(D) Venn diagram indicating the overlap among transcripts delimited by the red borders in (A)–(C).

(E) Sox17 transcripts are undetectable by RNA blotting in Fz4^{-/-} RECs (center) but are present in Fz4^{-/-} RECs following lentiviral transduction of Sox17 (right) at levels similar to those of WT RECs (left). The image is from one exposure of an RNA blot; for clarity, several lanes between lanes 2 and 3 have been removed.

(F) Lentivirus transduction of Sox17 rescues the ability of Fz4^{-/-} RECs to form capillary-like structures in Matrigel. Results are shown for two independent Fz4^{-/-} REC lines; the defective Matrigel behavior of the parental Fz4^{-/-} REC line 20 is shown in Figure 6C.

(Figures 7A–7C) led us to ask whether production of Sox17 alone might rescue the defect exhibited by Fz4^{-/-} RECs in Matrigel. To this end, multiple stable cell lines were generated by infecting Fz4^{-/-} REC lines with a lentivirus vector expressing Sox17. RNA blotting showed that Sox17 transcripts are readily detectable in WT RECs, undetectable in parental Fz4^{-/-} RECs, and present at roughly WT levels in the lentivirus-Sox17-transduced derivatives of Fz4^{-/-} RECs (Figure 7E). Matrigel assays with 11 cloned Sox17-transduced lines and pools of Sox17-transduced

cells derived from three Fz4^{-/-} REC lines revealed, in every case, a restoration of WT levels of capillary-like structures (Figure 7F). Thus, Sox17 appears to be a principal mediator of the angiogenic program controlled by Norrin/Fz4/Lrp signaling.

DISCUSSION

The principal findings of the present study are as follows: (1) Muller glial-derived Norrin acts on developing RECs and MCs

to control retinal vascular growth and architecture; (2) in the absence of intraretinal capillaries, communication from photoreceptors to RGCs is interrupted at the level of horizontal, bipolar, and/or amacrine cells, and this interruption is at least partially reversible; (3) Fz4 signaling regulates EC-MC interactions and is essential for vascular integrity in the retina and cerebellum; (4) Fz4 is present throughout the developing and adult vasculature, and its inappropriate activation results in severe vascular disorganization; (5) cultured *Fz4*^{-/-} RECs are unable to form capillary-like structures but can integrate into these structures when they are formed by WT RECs; (6) Norrin/Fz4/Lrp signaling controls a transcriptional program in endothelial cells that is, at least partially, mediated by Sox17; and (7) in vivo and in cell culture, Fz4 appears to be the principal—and likely the only—Norrin receptor. These results establish Norrin/Fz4/Lrp signaling as a central regulator of REC development, and they indicate that the hypovascularization responsible for Norrie disease, FEVR, and osteoporosis-pseudoglioma syndrome arises from the impairment of a specific transcriptional program. They also suggest that Fz4/Lrp signaling could play a role in vascular growth, remodeling, and maintenance in a variety of normal and pathologic contexts beyond the retina.

The Relationship between Retinal and Hyaloid Vasculatures

Our data distinguish between several competing hypotheses regarding the origins of retinal hypovascularization in *Fz4*^{-/-}, *Lrp5*^{-/-}, and *Ndp*⁻ mice (Kato et al., 2002; Ohlmann et al., 2004). In particular, the highly localized defects in vascular structure in mosaic *Fz4*^{CKOAP/-}; *Tie2-Cre* retinas and the failure of *Fz4*^{-/-} RECs to form capillary-like structures in vitro are inconsistent with the hypothesis that retinal hypovascularization in *Fz4*^{-/-}, *Lrp5*^{-/-}, and *Ndp*⁻ mice is secondary to inappropriate expansion and preservation of the hyaloid vasculature (a transient vascular structure within the vitreous cavity), which then increases retinal oxygenation and suppresses retinal vascularization. Instead, our data favor the opposite causal sequence: that the primary defect in *Fz4*^{-/-}, *Lrp5*^{-/-}, and *Ndp*⁻ mice is retinal hypovascularization, and the expansion and delayed regression of the hyaloid vasculature is a secondary consequence of retinal hypoxia. Our data also rule out models positing that the primary defect in Norrin/Fz4/Lrp signaling arises in RGCs, astrocytes, or other cells that guide vascular growth.

Control of Vascular Gene Regulation and Development by Norrin/Fz4/Lrp Signaling

The present study presents a genome-wide analysis of transcripts in the retinal vasculature, and it reveals several hundred transcripts with substantial changes in response to Norrin/Fz4 signaling, including Sox17 transcripts. In mice, Sox17 and Sox18 act in a partially redundant manner to promote angiogenesis (Matsui et al., 2006; Sakamoto et al., 2007), and Wnt/β-catenin signaling in differentiating ES cells leads to induction of Sox17 transcripts in developing endoderm and cardiac mesoderm (Liu et al., 2007). The demonstration that Sox17 rescues the Matrigel phenotype of *Fz4*^{-/-} RECs implies that Fz4/Lrp signaling in ECs activates a Sox17-dependent program that produces exploratory, migratory, and adhesive behaviors.

Using the *Ndp*^{AP} mouse we have demonstrated that *Ndp* is expressed principally in Muller glia. However, the ability of a Norrin transgene with lens-specific expression to rescue the *Ndp*⁻ retinal vascular phenotype argues that a precisely localized source is not essential for Norrin action (Ohlmann et al., 2005). Thus, Norrin/Fz4/Lrp signaling likely affects EC behavior at the level of cell competence rather than by acting directly as a chemo-attractant.

Minimal Overlap of Retinal Vascular Territories

The preservation of photoreceptor light responses and the loss of signal transmission in the inner retina in *Ndp*⁻ retinas (Luhmann et al., 2005b) and in *Fz4*^{-/-} and *Fz4*^{CKOAP/-}; *Tie2-Cre* retinas (this work) supports the existence of a sharp boundary between the territories served by the choroidal and retinal vasculatures, which supply the photoreceptors and the inner retina, respectively (Alm, 1992). Similarly, the normal functioning of intrinsically photosensitive RGCs in *Fz4*^{-/-} and *Fz4*^{CKOAP/-}; *Tie2-Cre* mice indicates a sharp boundary between the territories supplied by blood vessels at the vitreal face of the retina (RGC layer) and by intraretinal capillaries (inner nuclear layer). In *Fz4*^{-/-} retinas, defective signal transmission appears to arise from a partially reversible hypoxia, acidosis, or related metabolic derangement, rather than from irreversible damage. This finding implies that some neurons may be able to survive extended periods of hypo-perfusion, and that functional losses in some CNS vascular diseases, even over an extended time period, could be partially reversible.

Implications of Norrin/Fz4/Lrp Signaling for Retinal Vascular Diseases

It is intriguing to consider the possibility that Norrin/Fz4/Lrp signaling might be relevant to retinal vascular diseases such as diabetic retinopathy or retinopathy of prematurity. The continued expression of *Norrin*, *Fz4*, and *Lrp5* in the mature retina and the identification of *NDP* mutations in a few individuals with retinopathy of prematurity (Shastri et al., 1997) suggest that there may be a role for Norrin/Fz4/Lrp signaling in pathologic retinal vascular remodeling. Finally, the observations that some humans with *NDP* mutations exhibit peripheral venous insufficiency (Rehm et al., 1997; Michaelides et al., 2004), that Fz4 is expressed throughout the vasculature and is required for vascular integrity in the cerebellum (this study), that Fz4 and Norrin are required to maintain the capillaries of the stria vascularis (Rehm et al., 2002; Xu et al., 2004), and that Fz4 and Norrin are required for female fertility (Hsieh et al., 2005; Luhmann et al., 2005a) together suggest a wider role for this signaling system in vascular biology and disease.

EXPERIMENTAL PROCEDURES

Gene Targeting

The *Fz4*^{CKOAP}, *Z/Norrin*, *Ndp*⁻, and *Ndp*^{AP} alleles were created by standard gene targeting methods.

Microarray Hybridization

RNA samples were labeled with either the GeneChip Expression 3' Amplification One Cycle Target Labeling Kit (P/N 900493; for 1–5 μg RNA samples) or

the Ovation RNA amplification System V2 (P/N 3100; for ~0.1 μ g RNA samples) and FL-Ovation cDNA Biotin Module V2 (P/N 4200) kits (Nugen). The labeled probes were hybridized to Affymetrix mouse 430 2.0 chips, and microarray data were analyzed with Spotfire software.

Vascular Cell Purification and REC Culture

Immunopurification and cell culture were performed as described in Su et al. (2003).

Scratch Wound and Matrigel Assays

Scratch wounds were made by drawing a 1 ml pipette tip across the surface of a confluent REC monolayer. Matrigel assays were performed in DMEM with 1% FBS for 6–8 hr at 37°C. For mixed cultures, RECs were first incubated with 0.3% Dil or DiO.

Optokinetic Reflex and Pupillary Light Responses

OKR measurements were performed as described in Cahill and Nathans (2008). To measure pupil responses, the chamber was maintained in complete darkness for 30 s, followed by 30 s of constant bright light (400 lux).

Multielectrode Array Recordings

The multielectrode array (MEA) hardware and MCRack software (Multi Channel Systems, Reutlingen, Germany; distributed by ALA Scientific) and recording protocols are similar to those described by Meister et al. (1994).

Voltages were recorded from the 60 electrodes at 25,000 Hz and analyzed using MATLAB with routines based on the FIND 1.0 Toolbox (University of Freiburg; <http://find.bccn.uni-freiburg.de/>). Spikes were sorted by morphology using the K-means algorithm with 1, 2, 3, or 4 clusters, and for each K-means cluster at each electrode the peri-stimulus histograms for the 25 stimulus locations were calculated. Division into ON, OFF, and ON-OFF response classes was determined by calculating an ON versus OFF index based on the spike frequencies in the light and dark portions of the stimulus train [(spike frequency in light – spike frequency in darkness)/(spike frequency in light + spike frequency in darkness)]. The range of this index is –1 (exclusively OFF) to +1 (exclusively ON), and cells were categorized as ON, ON-OFF, or OFF if their index values were >0.3, between –0.3 and +0.3, or <–0.3, respectively.

ACCESSION NUMBERS

Microarray hybridization data have been deposited in the GEO database with accession code GSE16841.

SUPPLEMENTAL DATA

Supplemental Data include Supplemental Experimental Procedures, seven figures, four tables, and one movie and can be found with this article online at [http://www.cell.com/supplemental/S0092-8674\(09\)01034-4](http://www.cell.com/supplemental/S0092-8674(09)01034-4).

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